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The Na$^+$-coupled glucose transporter SGLT2 interacts with its accessory unit MAP17 *in vitro* and their expressions overlap in the renal proximal tubule

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Na$^+$-glucose cotransporter 2 is the renal Na$^+$-coupled glucose transporter responsible for the tubular glucose reabsorption, while MAP17 was recently identified as its accessory unit. Mutations in either of the proteins' coding genes, *SLC5A2* and *PDZK1IP1*, lead to urinary glucose excretion. To investigate whether MAP17 interacts with SGLT2 *in vitro*, we engineered a V5-tagged SGLT2 construct and evaluated HEK293T cells coexpressing it together with a HA tagged MAP17 construct. MAP17 is shown to colocalize and coimmunoprecipitate with SGLT2. Also, in human kidney sections, the expression of both proteins overlaps at the apical surface of tubular epithelia. This interaction provides the rationale behind SGLT2 activation by MAP17 as well the similarity of the *SLC5A2* and *PDZK1IP1* glucosuric phenotypes.

Keywords: genetics; glucose transport; renal; SGLT2

Two distinct members of the Na$^+$-coupled glucose transmembrane transporter family, SGLT1 and SGLT2, are responsible for the uptake of glucose from the proximal tubular lumen, the latter accounting for 97% of the glucose transport. Mutations in the SGLT2 coding gene, *SLC5A2*, cause familial renal glucosuria (FRG) [OMIM 233100], a codominant inherited phenotype characterized by the presence of glucosuria in the absence of generalized proximal tubule dysfunction or hyperglycemia [1].

The identification of SGLT1 [2] enabled the cloning of SGLT2 from a similar human kidney cDNA [3] that displayed distinct molecular and electrophysiological properties [4]. Selected as a candidate gene, *SLC5A2* was later found to underlie FRG [5].

Functionally, and compared to SGLT1, SGLT2 expresses poorly in transfected mammalian cells or *Xenopus laevis* oocytes and this fact has hampered a detailed characterization of its transport kinetics. Such a limitation was only circumvented by whole-cell patch-clamp techniques that have enabled the electrophysiological assessment of SGLT2 glucose transport [6]. Recently, MAP17 was identified by expression cloning as a SGLT2 β-subunit, while homozygous

Abbreviations
FRG, Familial Renal Glucosuria; HEK293T, human embryonic kidney cells; MAP17, 17 kDa membrane-associated protein; MDCK, Madin–Darby canine kidney cells; NHERF3, NHE3 regulatory factor 3; OK, opossum kidney cells; *PDZK1IP1*, PDZK1-interacting protein 1 (MAP17-coding gene); PDZK1, PDZ protein of kidney 1 (renamed NHERF3); PDZ, postsynaptic density protein PSD-95; SGLT2, Na$^+$-glucose cotransporter 2; *SLC5A2*, the SGLT2 solute carrier coding gene.

mutations in *PDZK1IP1* (MAP17 encoding gene) were found in one FRG individual negative for *SLC5A2* mutations [7]. In both mammalian cells [7] and oocytes [7,8], coexpression of MAP17 greatly increased SGLT2 transport activity and a comprehensive evaluation of SGLT2/MAP17 complex transport kinetics was achieved. It was found that glucose affinity for SGLT2, with a reported K_m of 3.4 mM, is ~10x lesser when compared to SGLT1 [8]. These findings validated earlier brush border membrane vesicle studies and microperfusion experiments (reviewed in [9]) as well observations in the *Sglt2*-deficient mice [10], that predicted an active glucose transport in the early S1 proximal tubule *via* a low-affinity/high-capacity transporter (SGLT2/MAP17), while in the later S3 segment, a high-affinity/low-capacity system would predominate (SGLT1). Therefore, the differences in expression patterns and transport kinetics of both systems along the proximal tubule act in synergy to minimize glucose loss in the urine.

The way MAP17 and SGLT2 interact is not clear. Immunofluorescence and Western experiments with OK cells cotransfected with tagged constructs for both proteins, did not reveal an increase in SGLT2 cell surface expression [7]. This lead the authors to hypothesize that MAP17 could induce a SGLT2 conformational change, responsible for the observed cotransporter increased activity.

However, an interaction between both proteins has never been explored. In order to do so, we have used two different cellular models expressing SGLT2 and MAP17 constructs, tagged with, respectively, V5 and HA epitopes, and assessed their interaction *in vitro* by immunofluorescence and immunochemistry techniques using anti-V5 and -HA antibodies. We first engineered a V5-tagged SGLT2 construct and produced a cell line that stably expressed the construct, with the purpose of minimizing cellular protein overloading in colocalization studies and, hence, reproduce a more physiological level of protein abundance. This cell line was subsequently transiently transfected with the HA-tagged MAP17 construct, and subcellular localization of both proteins was assessed by confocal microscopy. Also, HEK293T wild-type cells were cotransfected with both tagged constructs, aiming at a transient overexpression of both proteins, and were evaluated by immunocytochemistry as well as coimmunoprecipitation assays. Additionally, we performed immunofluorescence labeling of MAP17 and SGLT2 in paraffin-embebbed kidney biopsies with no detectable histopathological changes with antipeptide antibodies for MAP17 and SGLT2.

Materials and methods

Plasmid construction

We purchased the human SGLT2 cDNA cloned in a pEZ-M29 plasmid displaying an EGFP N-terminal tag (Genecopoeia, clone EX-C0047-M29) and subsequently sequenced it with internal primers in order to verify the identity with Genebank accession NM_003041. The SGLT2 cDNA insert was long-range PCR amplified with the following sense, ATTGAATTCAACCATGGAGGAGCA, and reverse, TATAAGCTTAGGCATAGAAGCCCC, primers that introduced new restriction sites, and then ligated to an empty pcDNA3.1 plasmid after double *EcoR*I/*Hind*III digestion of, both, the insert and vector, originating the pcDNA3.1 SGLT2. The insert was again sequence verified and directly cloned into the pcDNA ENTR BP V5 by digesting the insert with *EcoR*I/*Pme*I and the vector with *EcoR*I/*EcoR*V. The pcDNA ENTR BP V5 had been previously generated based on the pcDNA ENTR BP GFP [11], a mammalian expression Gateway® plasmid (Invitrogen, Carlsbad, CA, USA), by swapping the GFP with the V5 epitope and a chemically synthesized polylinker. The V5 epitope and the polylinker were digested with *Nhe*I/*BamH*I and cloned in the vector previously digested with the same set of restriction enzymes. This pcDNA ENTR BP V5-SGLT2 construct has, therefore, an engineered N-terminal V5 and was created with the ultimate goal of mammalian cell line transduction using a pLenti6-DEST gateway vector expression system. The pcDNA ENTR BP V5-SGLT2 was also sequence verified before heterologous expression studies.

The plasmid containing the human MAP17 with a HA epitope inserted at residue 23, referred as pcDNA3.1 HA23MAP17, was a kind gift from M. Coady (Montreal, CA, USA).

Cell culture, transient transfection, and stable cell line production

The HEK293T cells were grown in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS and 5% penicillin–streptomycin antibiotic solution. For stable cell line production, HEK293T cells were transfected with pLenti V5-SGLT2 plasmid using Jetprime DNA-siRNA transfection reagent (Polyplus, Illkirch, France). Lentiviral particles were collected after 4 days and stored at −80 °C. These were used to transduce HEK293T cells for 24–48 h; cells were then positively selected for 1 week with 8 $\mu g \cdot mL^{-1}$ blasticidine (Invitrogen), followed by clone selection. This cell line will be referred throughout the text as HEK293T V5-SGLT2.

For immunofluorescence experiments, cells were seeded on glass coverslips in 12-well plates and either cotransfected (for HEK293T wild-type) with a total of 400 ηg of pcDNA ENTR BP V5-SGLT2 and pcDNA3.1 HA23MAP17 DNA,

or transfected (for HEK293T V5-SGLT2) with 200 ηg of the latter, using the Jetprime DNA-siRNA transfection reagent. For western blot or coimmunoprecipitation, 60-mm Petri dishes and 2 μg of DNA were used instead. Cells were transfected at 40–50% confluence and then harvested for various analyses at 24–48 h post transfection.

In order to validate heterologous expression of single transfection experiments and evaluate anti-SGLT2 and -MAP 17 antibodies specificity, we compared nontransfected and single transfection experiments with either pcDNA ENTR BP V5-SGLT2 or pcDNA3.1 HA23MAP17, this time using 200 ηg and 1 μg of DNA for immunofluorescence experiments and western blot analysis, respectively.

Immunofluorescence analysis of cell lines

Transfected HEK293T or HEK293T V5-SGLT2 cells were grown on glass coverslips for 24–48 h after transfection, then washed with PBS and fixed in 4% formaldehyde in PBS at room temperature (RT) for 10 min, washed again and permeabilized with a solution of Triton X-100 0.25% in PBS for 10 min at RT. Samples were blocked with 3% BSA in PBS for 30 min at RT, then incubated with rabbit anti-HA rhodamine TRITC conjugate antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-805) at 1 : 20 dilution in 1% BSA in PBS for 2 h at RT, after which they were washed and incubated overnight at 4 °C with a goat anti-V5 epitope primary antibody (Sicgene, Coimbra, Portugal #AB0096-500) at 1 : 1000 dilution in 1% BSA in PBS. The following day, cells were incubated with donkey anti-goat IgG Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA #A-11055) for 45 min at RT, 1 : 800 dilution in 1% BSA in PBS. After additional washing, cells were incubated for 8 min at RT with a Hoechst (Chemicon International, Temecula, CA, USA) solution of 0.25 $\mu g \cdot mL^{-1}$ in water and finally mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunolabeling was evaluated with a confocal LSM710 microscope using the ZEN software (all Carl Zeiss GmbH, Darmstadt, Germany).

In single-transfection experiments, for assessing heterologous expression of cellular systems and anti-SGLT2 or -MAP17 antibody specificities, similar protocols were used. However, additional goat primary antibodies against peptides from the human proteins SGLT2 (Santa Cruz Biotechnology #sc-47402) and MAP17 (Santa Cruz Biotechnology #sc-27376) were used, both at 1 : 60 dilution.

Western blot analysis of cell lysates

Whole-cell lysates were recovered from 60-mm Petri dish samples by adding 300 μL of 1X Laemmil reducing buffer, followed by continuous gentle agitation for 10 min on ice, and, once recovered in a 2-mL polypropylene tube, submitted to brief sonication. For SGLT2 immunodetection, 10–20 μL of protein extracts were immediately separated by SDS/PAGE electrophoresis in a 10% acrylamide gel and transferred onto nitrocellulose membranes at 75 V constant for 90 min, while for MAP17, samples were heated to 95 °C for 10 min previous to SDS/PAGE electrophoresis in a 12.5% gel and further transferred onto nitrocellulose membranes at 75 V constant for 45 min.

Membranes were incubated for 1 h at RT in blocking buffer (2% egg albumin in PBS-Tween 0.01%), washed twice in PBS-Tween 0.01% and incubated at 4 °C overnight in PBS-Tween 0.01% with the following primary goat antibodies (and dilutions): anti-V5 (1 : 2500), anti-HA (1 : 1000), anti-SGLT2 (1 : 500), and anti-MAP17 (1 : 500). For western blot analysis of coimmunoprecipitation beads' elution, mouse anti-V5 (Invitrogen #46-0705; 1 : 2000) and mouse anti-HA (Thermo Fisher Scientific #26183; 1 : 500) primary antibodies were used instead of goat antibodies. After washing with PBS-Tween 0.01%, the membranes were incubated for 45 min at RT with horseradish-coupled secondary antibodies against goat (Thermo Fisher Scientific #A2446) or mouse (Invitrogen #67-652) IgG in 1% egg albumin blocking solution. The western blot was developed using a chemiluminescent detection reagent (GE Healthcare, Chicago, IL, USA) and image acquisition performed in a ChemiDoc Touch Image system and analyzed using the IMAGE LAB 5.2 software (all Bio-Rad, Hercules, CA, USA).

Coimmunoprecipitation

Cell lysates were prepared using a mixture of 2% of CHAPS (BioChemica, Darmstadt, Germany), NP-40 (Calbiochem, La Jolla, CA, USA), and Triton X-100 (Sigma, St. Louis, MO, USA) in lysis buffer (150 mM NaCl and 50 mM Tris-HCl pH 7.4), since SGLT2, a predicted 14 transmembrane domain protein, was found difficult to elute from membrane fractions if only 1% CHAPS was being used. This was the reason, too, on why we were not able to recover enough SGLT2 in the lysate supernatant from the HEK293T V5-SGLT2 cell line and, therefore, restricted our coimmunoprecipitation experiments to cotransfected cells overexpressing the SGLT2 construct.

Protein lysates were recovered from transfected (pcDNA3.1 HA23MAP17 for HEK293T V5-SGLT2) and cotransfected (pcDNA ENTR BP V5-SGLT2 and pcDNA3.1 HA23MAP17 for HEK293T wild-type) cells grown in 60-mm Petri dishes, by incubating the samples with 300 μL of 2% lysis buffer supplemented with an in-house prepared protease inhibitor cocktail, under gentle agitation for 10 min on ice. Once transferred to a 2-mL polypropylene tube, samples were further incubated on ice for 2 h while being vortexed and homogenized every 15 min and, finally, centrifuged 17 000 ***g*** for 20 min at 4 °C. V5-SGLT2 immunoprecipitation was performed by incubating overnight, at 4 °C, the lysate supernatant with 20 μg of goat anti-V5 antibody in a 0.5% lysis buffer, and, in the following night, with protein G sepharose beads

(Invitrogen). The beads were then rinsed three times with lysis buffer and proteins eluted in 70 μL of 1X Laemmil loading buffer, heated to 50 °C for 10 min. Subsequently, samples were processed for immunoblotting. Findings were replicated in two independent experiments.

Western blotting, histological examination, and immunofluorescence of kidney specimen

Membrane protein fractions from surgical biopsy samples of nontumor-involved areas of nephrectomy specimen were recovered using the ProteoJET protein extraction kit (Thermo Fisher Scentific) according to manufacturer's instructions and frozen at −80 °C until further analysis. Twenty micrograms of protein was applied in each lane and immunoblotted with goat primary antipeptide antibodies against human SGLT2 and MAP17 as detailed above. For immunofluorescence purposes, formalin-fixed and paraffin-embedded kidney biopsies showing no detectable histopathological changes were evaluated. Heat-induced epitope retrieval was applied to 3-μm consecutive sections of the specimen with Target Retrieval Solution pH 9 (Dako, Santa Clara, CA, USA) at 95–99 °C for 20 min. Incubations with goat primary antibodies anti-human MAP17, at 1 : 20 dilution, and anti-human SGLT2, at 1 : 5 dilution, as well donkey anti-(goat IgG) Alexa Fluor 488 secondary antibody, at 1 : 200 dilution, were all carried out for 1 h at RT. An epifluorescence Zeiss Axio Imager Z2 microscope was used for image acquisition and analysis, using the ZEN software. And since both primary antibodies originated from goat, colocalization experiments were not carried out. Instead, we used topology references across sections to evaluate the extension of overlap in proteins' expression.

Results

Western blotting of whole-cell lysates from transfection experiments

To confirm that SGLT2- and MAP17-tagged proteins were being properly expressed with the expected molecular weight in our cellular models and, simultaneously, to evaluate primary antibody specificity against tags (V5, HA) or the human proteins (SGLT2, MAP17), we studied HEK293T cells transfected with the different plasmid constructs by immunofluorescence (Fig. S1) and western blotting of whole-cell lysates (Fig. 1). For the pcDNA ENTR BP V5-SGLT2 plasmid construct, both anti-SGLT2 (Fig. 1A lane 3) and anti-V5 (Fig. 1A lane 6) antibodies identified a broad band of 65 kDa in samples from transfected cells and that was slightly larger than the one seen with cells transfected with pcDNA3.1

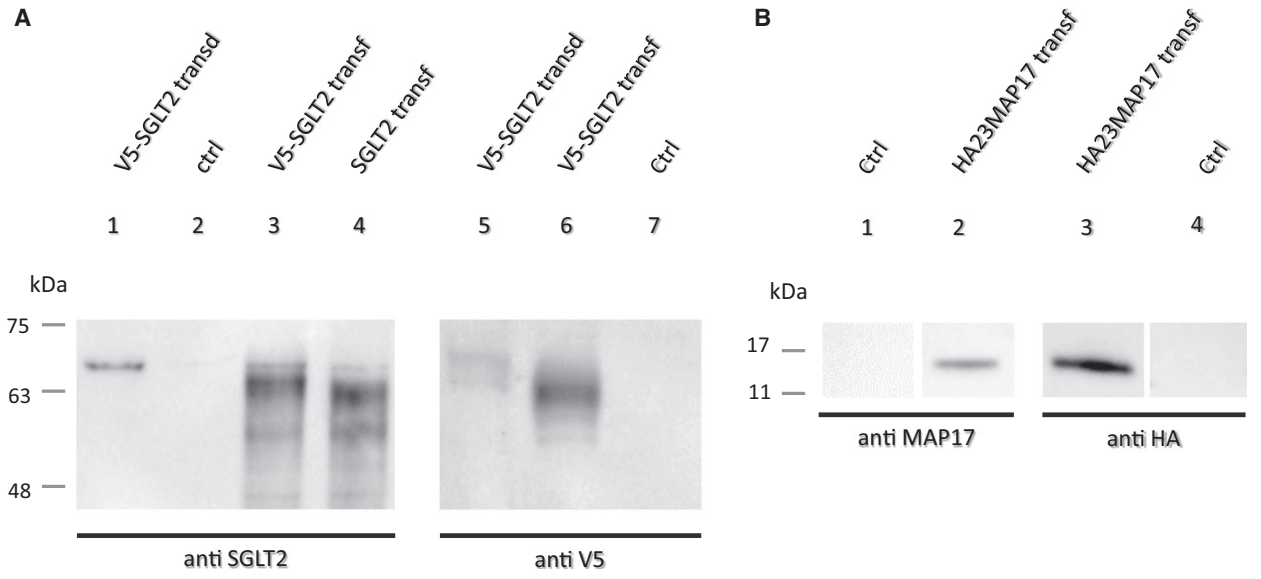

Fig. 1. Western blot analysis of heterologous expression of SGLT2 and MAP17 constructs in HEK293T cells. (A) Western blot of SGLT2 constructs: immunoblotting of samples transfected with the V5 construct, using either anti-SGLT2 (lane 3) or anti-V5 (lane 6) antibodies, revealed a broad band of ~65 kDa that is slightly larger than the one observed for the untagged protein (lane 4) probed with anti-SGLT2 antibody. In HEK293T transduced with the lentivirus expressing the V5-SGLT2 construct, a similar band is observed, either with anti-SGLT2 (lane 1) or anti-V5 (lane 5). (B) Western blot of the HA23MAP17 construct reveals the same ~15 kDa band when immunoblotted with either anti-MAP17 (lane 2) or anti-HA (lane 3) antibodies. V5-SGLT2 transd: HEK293T transduced with the lentivirus expressing V5-SGLT2 construct; V5-SGLT2 transf: HEK293T-transfected with the V5-SGLT2 construct; SGLT2 transf: HEK293T transfected with the untagged SGLT2 construct; HA23MAP17 transf: HEK293T transfected with the HA23MAP17 construct; ctrl: nontransfected HEK293T wild-type cells used as controls.

SGLT2 and immunoblotted with the anti-SGLT2 antibody (Fig. 1A lane 4). This finding is in accordance with the V5 N-terminal modification of SGLT2 in the pcDNA ENTR BP V5-SGLT2 construct. In the HEK293T V5-SGLT2 cell line that stably expressed the V5-SGLT2 construct, a band of similar molecular weight was also detected with anti-SGLT2 (Fig. 1A lane 1) or anti-V5 (Fig. 1A lane 5) antibodies, although, for the latter, an additional and close together smaller band, was also observed. For the transfection experiments with pcDNA3.1 HA23MAP17 plasmid, immunoblotting with either anti-MAP17 or anti-HA, gave rise to a similar band of 15 kDa in transfected cells (Fig. 1B, lanes 2 and 3). Therefore, we confirmed that the engineered constructs lead to mammalian cell expression of SGLT2 or MAP17 at the expected molecular weight and that antibodies against V5 or HA tag epitopes and SGLT2 or MAP17 are similarly specific in these whole cell lysates from heterologous expression experiments.

MAP17 colocalizes with SGLT2

To initially test the hypothesis that MAP17 and SGLT2 interact, we cotransfected HEK293T with the tagged SGLT2 and MAP17 constructs and evaluate their subcellular localization. Similar assays were performed with the HEK293T V5-SGLT2 cell line stably expressing the V5-SGLT2 construct and transfected with pcDNA3.1 HA23MAP17 plasmid. By confocal microscopy, and after dual immunolabeling, MAP17 was shown to colocalize with SGLT2 in both HEK293T cell lines (Fig. 2). In addition to a very strong perinuclear staining, the observed vesicular pattern is compatible with the localization of proteins within lipid rafts. HEK293T are not polarizable cells and so, a clear membrane cell surface expression is not expected to occur.

MAP17 coimmunoprecipitates with SGLT2

The colocalization of both proteins provided the first clue that MAP17 and SGLT2 could interact physically. To further explore the *in vitro* interaction of both proteins, coimmunoprecipitation experiments were performed (Fig. 3). HEK293T cells were either transfected with pcDNA3.1 HA23MAP17 alone, or cotransfected with pcDNA3.1 HA23MAP17 and pcDNA ENTR BP V5-SGLT2 plasmids. Coimmunoprecipitation of

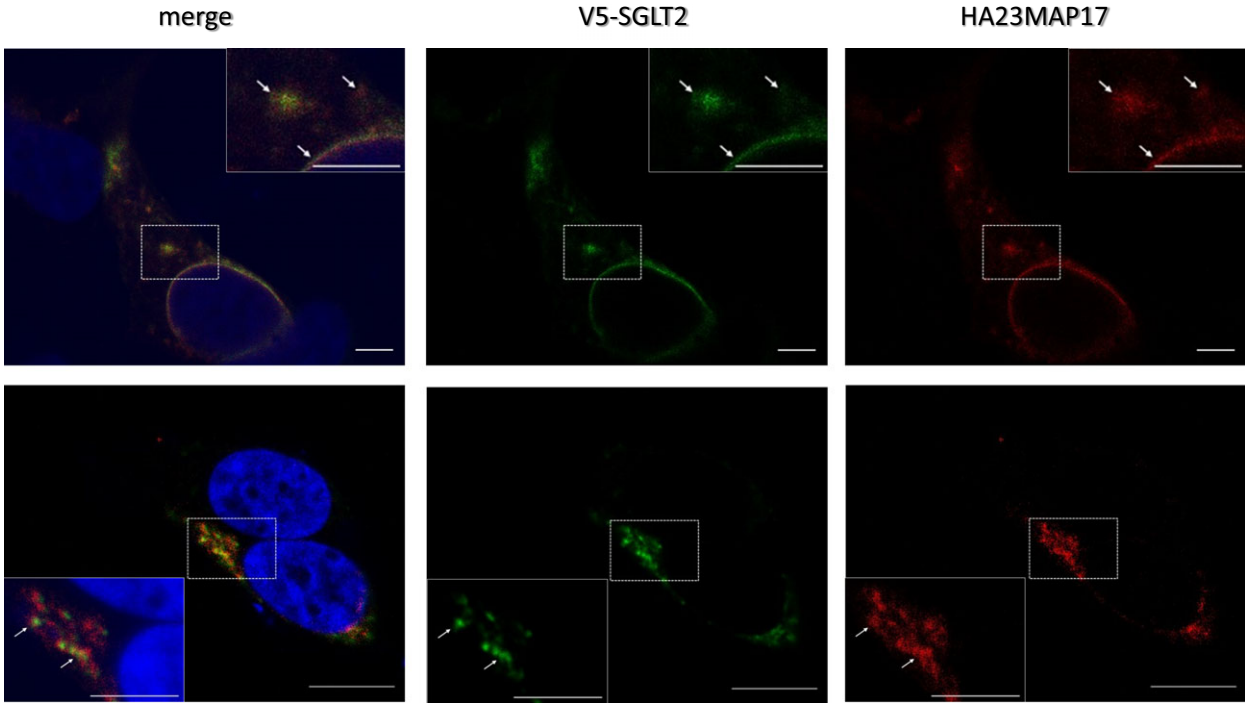

Fig. 2. Confocal immunofluorescence imaging of representative X-Y planes of heterologous cellular systems expressing SGLT2 and MAP17 constructs. Top panel: cotransfected cells with the V5-tagged SGLT2 and the HA-tagged MAP17 constructs. Lower panel: HEK293T transduced with lentivirus expressing the V5-tagged SGLT2 construct that were transfected with the HA-tagged MAP17 coding plasmid. MAP17 is seen to colocalize with SGLT2 (arrows) in intense perinuclear and vesicular patterns. Incubation was performed with rabbit anti-rhodamine TRITC conjugate antibody followed by goat anti-V5 primary and donkey anti-goat IgG Alexa Fluor 488 secondary antibodies, as described in material and methods. Nuclear DNA stained by Hoechst. Scale bar, 5 μm.

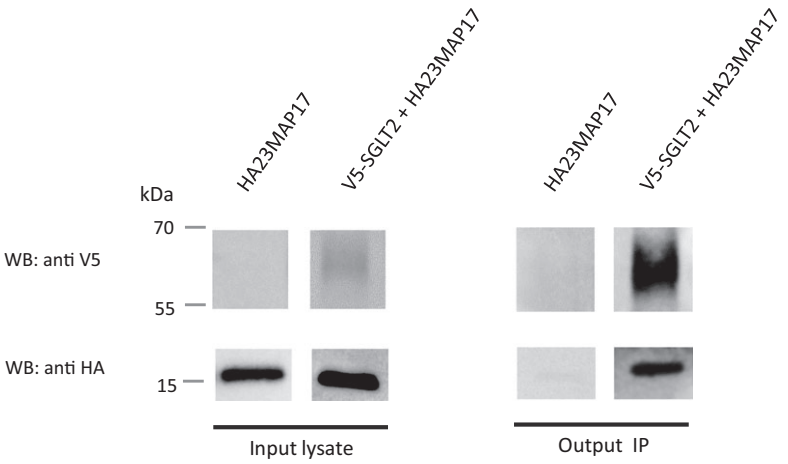

Fig. 3. *In vitro* interaction between SGLT2 and MAP17. HA23MAP17 is present in lysates (input) of cells transfected with pcDNA3.1 HA23MAP17 or cotransfected with pcDNA ENTR BP V5-SGLT2 and pcDNA3.1 HA23MAP17. However, only in the presence of V5-SGLT2 was HA23MAP17 coimmunoprecipitated by goat anti-V5 antibody, as detected in the output using mouse anti-HA antibody. WB, western blot; IP, immunoprecipitate.

V5-tagged SGLT2 was performed with goat anti-V5 antibody in the presence of protein G sepharose beads. Western blot analysis of eluted proteins with mouse anti-V5 and HA immunoblotting identified the HA-tagged MAP17 construct in eluted proteins originating from lysate supernatant of cotransfected samples, but not when MAP17 was being overexpressed alone, providing evidence that SGLT2 and MAP17 do interact with each other *in vitro*.

MAP17 and SGLT2 overlap their expression in human normal kidney

We extended our observations to normal human kidney biopsies and assessed the expression of both proteins. Specificity of the antipeptide human SGLT2 and MAP17 antibodies was assessed by western blotting (Fig. 1) or immunocytochemistry (Fig. S1) analysis of HEK293T transfected and transduced with the different cDNA constructs. In addition, western blotting of protein membrane fractions of normal human kidney incubated with the anti-human SGLT2 and MAP17 antibodies, revealed the expected bands of 75 and 17 kDa, respectively (Fig. 4). For SGLT2, a less intense band of an estimated molecular weight of 135 kDa was also identified, probably resulting from SGLT multimerization reported to occur when frozen samples are used [9]. The western blot analysis of SGLT2 in human kidney now presented, replicate our previous findings that have been published in the abstract form (Aires I, Santos AR, Genc G, Bogarin R, Calado JT. Glucose transport in the proximal tubule: disruption of urate transport in familial renal glucosuria and report on SGLT2 expression in normal and pathological kidney [Abstract] (2012) *J Am Soc Nephrol* 23, 730A). Finally, in the formalin-fixed and paraffin-embedded specimen, we found that SGLT2 and MAP17 largely overlap their expression (Fig. 4). Specifically, both displayed similar topological expression at the brush border of proximal tubule epithelial cells.

Discussion

Na^+-glucose cotransporter 2 is a predicted 14 transmembrane domain Na^+-coupled glucose transporter that accounts for the bulk of glucose reabsorption in the proximal tubule and, as such, mutations in its coding gene are responsible for the large majority of FRG cases [5]. Recently, SGLT2 was selected as a therapeutic target for the treatment of type 2 diabetes *mellitus* based on the rational that SGLT2 pharmacological inhibition would lead to a significant reduction in the renal threshold for glucose excretion and, therefore, promote urinary disposal of the excess of glucose [12]. In spite of the fact that hundreds of thousands of diabetic individuals are currently taking SGLT2 inhibitors, little is known about SGLT2 post-transcriptional regulation. The finding, by means of expression cloning, that MAP17 is an accessory unit for the activity of SGLT2 provided the first hint on such a regulation [7].

MAP17, a two-transmembrane protein, was first cloned by differential display techniques as a protein upregulated in several human carcinomas [13]. In normal tissues, however, it is only significantly expressed in the human kidney, where it locates to the brush border of proximal tubular cells [13]. Further insights on the role of MAP17 in the proximal tubule were revealed by two-hybrid experiments that have used PDZ domains from the PDZK1 protein as baits for an adult mouse kidney cDNA library, and through which MAP17 was identified as the most likely candidate to

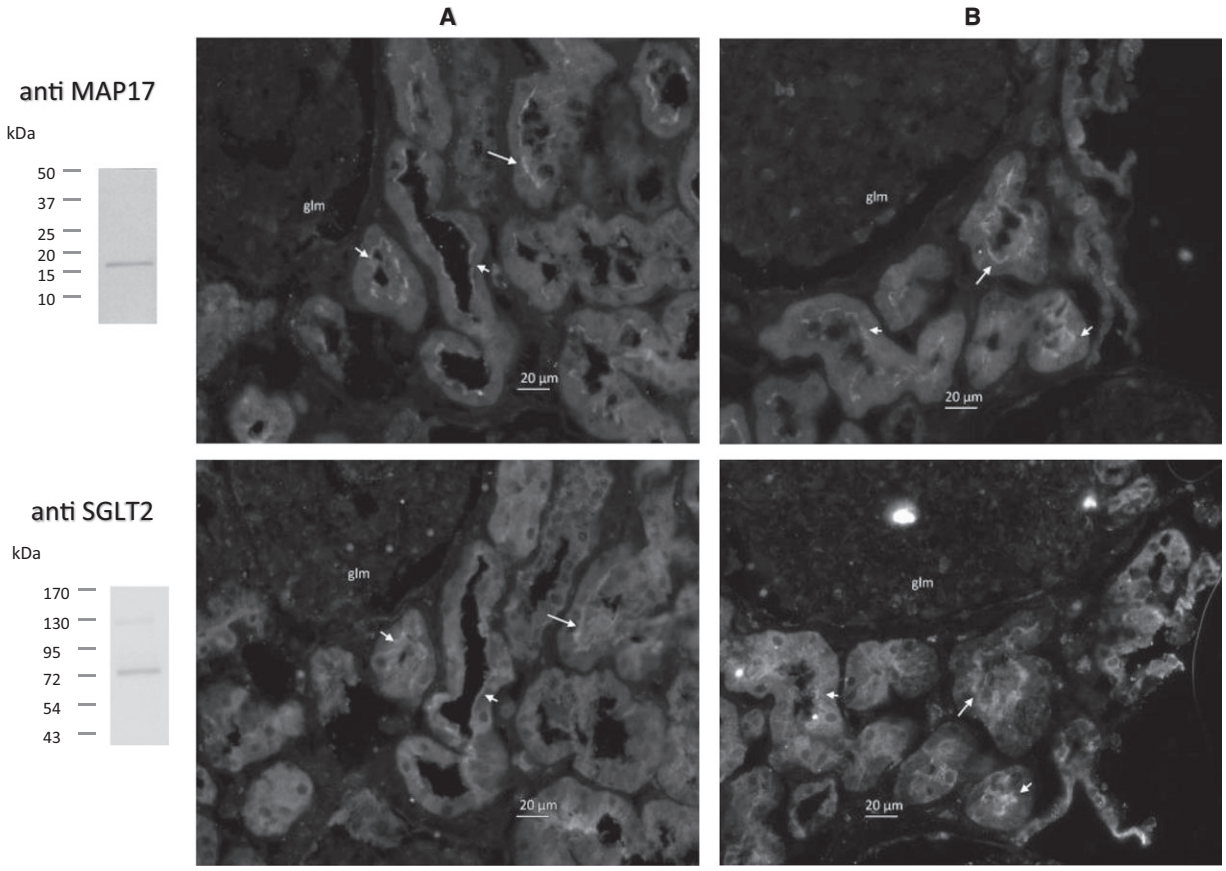

Fig. 4. Constitutive SGLT2 and MAP17 expression in the human kidney. Consecutive kidney sections immunolabeled with goat anti-MAP17 (top panel) or SGLT2 (bottom panel) antibodies are shown. For each antibody, the immunoblotting of protein extracts from human normal kidney is also depicted. In two distinct areas of the cortex (A and B), both proteins are shown to largely overlap their expression in the apical surface of epithelial cells of proximal tubules. For each area, the same specific arrow (of a given size and direction) is used to point out, across sections, the tubule segments simultaneously expressing MAP17 and SGLT2. Donkey anti-(goat IgG) Alexa Fluor 488 was used as a secondary antibody, as described in material and methods. Glm, glomerulus; scale bar, 20 µm.

interact with PDZK1 [14]. PDZK1 (now renamed NHERF3), a member of a large family of PDZ proteins, is the major scaffolder in the brush border, coordinating the abundance of transport and signaling proteins like NaPi-IIa, NH3, CFEX, URAT1, or PTHR1, all displaying a COOH-terminus PDZ recognition consensus sequence [14]. Additionally, the NH_2 terminus of NaPi-IIa interacts with MAP17 and both proteins are expressed within the S1 segment of the proximal tubule while overlapping in their localization to the brush border [15]. It was also shown that the coexpression of MAP17 and PDZK1 induced internalization of NaPi-IIa, therefore postulating a role for the heterotrimeric complex in phosphate reabsorption [16].

In a similar manner, our current work aimed at providing evidence that MAP17 interaction with SGLT2 underlies the reported increased transport activity of the SGLT2/MAP17 complex. We designed two heterologous cellular expression models to test for an *in vitro* interaction: one, based on a lentivirus-transduced V5-tagged SGLT2 construct that we have engineered, followed by transfection with the HA-tagged MAP17 construct; the other, on the simultaneous cotransfection of both constructs. The major advantage of the former model is to minimize SGLT2 protein cell overloading that could limit the interpretation of colocalization studies. Due to the uncertainty regarding the specificity of the commercially available antipeptide antibodies against SGLT2 or MAP17, anti-V5 and -HA epitopes antibodies were used, instead, as reference. The expected molecular weight for SGLT2 is 70–75 kDa which has been verified in several reports using kidney tissue [9,10,17] although it can vary with the degree of glycosylation, depending on the species or cell type [9]. Western blotting of human kidney samples incubated with the antibody

against SGLT2 used in the current study revealed a band of 75 kDa. But our SGLT2 constructs, when overexpressed in HEK293T cells, displayed a molecular weight 65 kDa, regardless of whether we were using antibodies against V5 or SGLT2. The difference in the observed molecular weight may be caused by incomplete denaturation of multiple-pass transmembrane proteins by SDS [9] and has been reported already in a HA-tagged SGLT2 construct also overexpressed in HEK293T cells and blotted with an anti-HA antibody [18]. In our experiments, SGLT2 immunoblotting was performed immediately after recovery of cell lysates and using nonheated samples, since, similarly to what was described for other transmembrane proteins [19], denaturation greatly impaired SGLT2 immunodetection. This fact, coupled with the complexity of membrane protein glycosylation seen in whole-cell lysates, is responsible for the multiple band pattern observed in transfected cells. However, it cannot account for the discrepancy observed between transduced cells immunoblotted with anti-SGLT2 (Fig. 1A lane 1) and anti-V5 (Fig. 1A lane 5) antibodies, displaying, respectively, one or two closely located bands. We can presume that some degree of proteolytic degradation may have occurred, affecting the epitope recognized by the anti-SGLT2 antibody. Accordingly, anti-V5 (at the NH2 terminus of the construct) recognizes the full length as well the partially degraded protein, while the anti-SGLT2 only identifies the nondegraded form. In transfected cells this may have been unnoticed due to the higher level of protein expression.

In both HEK293T cell lines (wild-type and V5-SGLT2), heterologous expression revealed that MAP17 colocalizes with SGLT2 in a perinuclear and vesicular pattern. HEK293T are not polarizable cells and so, the lack of clear surface expression was not unexpected. Previous work on similarly transfected HEK293T cells detailing the kinetics of glucose transport and using radiotracer uptake and patch-clamp techniques, provides strong evidence that in HEK293T overexpressing SGLT2, an adequate amount of the transporter is inserted in the membrane of these cells [6]. It is unfortunate that, in spite our best efforts, we did not succeed in developing in the more suitable OK or MDCK cell lines, a clone stably expressing the V5-tagged SGLT2 protein. In line with the colocalization experiments, our coimmunoprecipitation assays using the HEK293T cell line cotransfected with the SGLT2- and MAP17-tagged constructs, revealed that MAP17 coimmunoprecipitated with anti-V5 antibodies, only if the V5-tagged SGLT2 construct was simultaneously being overexpressed. These findings further strengthen the hypothesis that, at least in these cellular models, both proteins interact. Still, the approach used in the current work cannot unravel the nature of this interaction. Namely, we cannot confirm if MAP17 is a direct binding partner of SGLT2 or, alternatively, this interaction is mediated by additional proteins constitutively expressed in HEK293T cells, nor can we identify the protein domains involved.

Contrary to PDZ-interacting proteins like NaPi-IIa, SGLT2 displays no COOH-terminus PDZ recognition consensus sequence and accordingly, transport activity of the SGLT2/MAP17 complex was not impaired when SGLT2 was coexpressed with a MAP17 construct lacking the terminal PDZ recognition consensus sequence STPM (MAP17ΔSTPM) instead of the full-length protein, revealing that the heteromeric complex is fully functional in the absence of PDZK1 [7]. Also, the *Pdzk1* knockout mouse has been published and while urinary glucose excretion was not specifically evaluated, compared to wild type, there was no difference in plasma glucose concentration [20]. NaPi-IIa/MAP17 interaction was identified by two-hybrid experiments but never confirmed by the *in vitro* interaction assays performed, leading to the conclusion that this was an interaction of a weak nature that could only be stabilized within the MAP17/NaPi-IIa/PDZK1 complex [15]. Our coimmunoprecipitation results postulate, instead, a strong interaction between SGLT2 and MAP17, a fact that could obviate PDZK1 stabilization, in agreement with the above-mentioned transport experiments performed with SGLT2/MAP17ΔSTPM.

Finally, we have shown that SGLT2 and MAP17 overlap their expression at the apical surface of proximal tubule epithelial cells in the human kidney. Previous reports have documented, separately, for SGLT2 [21] or MAP17 [13], that these proteins are expressed at the brush border of the proximal tubule epithelial cells of the human kidney. We now provide evidence that their expression overlaps along similar segments of the proximal tubule. Having performed the immunofluorescence staining of human kidney sections with available commercial antibodies, and aware that, for SGLT2 in particular, there are few well-characterized antibodies [17,22,23], we validated the purchased anti-SGLT2 and -MAP17 antibodies in heterologous expression systems, by crosschecking them with the anti-tag antibodies used as reference, and also by immunoblotting with protein extracts from normal human kidney.

In summary, we here provide evidence that SGLT2 and MAP17 when overexpressed interact *in vitro* and, likewise, under constitutive expression in the kidney they also overlap their expression in the proximal

tubule. This interaction may provide the rationale behind the SGLT2-mediated glucose transport activation by MAP17 as well the similarity of the *SLC5A2* and *PDZK1IP1* glucosuric phenotypes.

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Author contributions

JC designed, performed and evaluated the experiments as well drafted the manuscript; ARS performed the histological and immunofluorescence experiments in kidney specimen; IA performed the sub-cloning experiments; FL performed the western blotting; FN evaluated the kidney histological and immunofluorescence findings; JR designed the experiments, reviewed and approved the manuscript; JR designed the experiments, performed the sub-cloning experiments, reviewed and approved the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Validation of MAP17 and SGLT2 antipeptide antibodies in heterologous expression systems by immunofluorescence.